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(54) Title: METHODS AND COMPOSITIONS FOR IMPROVING THE DEVELOPMENTAL ABILITY AND QUALITY OF PRE-IMPLANTATION EMBRYOS

(57) Abstract

The present invention provides a method for improving the developmental ability and quality of pre-implantation embryos by partially inhibiting the ability of the embryos to generated ATP by oxidative phosphorylation in *in vitro* culture.

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METHODS AND COMPOSITIONS FOR IMPROVING THE DEVELOPMENTAL ABILITY AND QUALITY OF PRE-IMPLANTATION EMBRYOS

Field of the Invention

The present invention relates to methods and compositions for improving the developmental ability and quality of pre-implantation embryos.

Background of the Invention

The metabolism of pre-implantation embryos has been studied in a number of mammalian species. As with most cells, pre-elongation embryos, for example bovine pre-elongation embryos, are highly dependent on oxidative phosphorylation as the primary energy production pathway (i.e. ATP generating pathway) (Thompson et al. 1996). This is particularly so during pre-compaction development, where it is estimated that approximately 90-95% of all ATP is derived from oxidation (Thompson et al. 1996). During compaction and blastulation, the demand for ATP increases, to allow increases in protein synthesis (Thompson et al. 1998) and the activity of the Na+/K+-ATPase (Leese 1991), which forms an osmotic potential across the trophectoderm, producing the blastocoel cavity. The increased demand for ATP causes increases in consumption of the major substrates, including oxygen and pyruvate (Thompson et al. 1996), amino acids (Partridge et al. 1996) and glucose (Thompson et al. 1996). However, most of the glucose that is metabolised by ruminant embryos at the blastocyst stage is accounted for by glycolysis, with lactate as the end-product and is transported from the embryo to the surrounding medium. Little glucose is oxidised - what is, is mostly via. the pentose-phosphate pathway for ribose formation, rather than by the tri-carboxylic acid cycle (Gardner et al. 1993; Rieger and Guay 1988; Thompson et al. 1991, 1996). However, the increase in glucose consumption is such that the contribution of glycolysis alone to ATP production was

measured to increase from approximately 4-8% to 15-18% between pre- and post compaction stages, respectively, in an environment in which O_2 was abundant.

Several studies have suggested that the O_2 tension of the reproductive tract decreases as embryos pass from the ampulla of the oviduct to the uterine cavity (Brown and Mattner 1984; Fischer and Bavister 1993). Furthermore, of the little information we have concerning the metabolism of ICM-cell tissue, it would appear that this is more glycolytic in activity than that of the surrounding trophectoderm (Hewitson and Leese 1993). These data suggest that there is a shift in the metabolic pathway preference for embryonic ATP production from oxidative phosphorylation to glycolysis, to correspond with development within the uterine cavity, an environment in which O_2 availability may well be limited.

Such a shift in pathway preference is not unknown for mammalian embryos. Both the human (Gott et al. 1990) and rat embryos (Brison and Leese 1991) appear to behave similarly to the ruminant embryo in this manner. The mouse embryo, the most examined model of early embryo development, is totally dependent on oxidative phosphorylation throughout development, but changes substrate preference with compaction from that of the tri-carboxylic acids to glucose (Leese 1991). In this species, oxidation of glucose following compaction occurs readily and glucose provides essential energy requirements during this period of development (Leese 1991).

Using the rat embryo as a model, Brison and Leese (1994) demonstrated that embryos were not reliant on oxidative phosphorylation during compaction and blastulation. These authors used several oxidative phosphorylation uncouplers or inhibitors (e.g. 2,4-dinitrophenol or cyanide, respectively) to demonstrate that blastulation can take place *in vitro* in the presence of these compounds. Furthermore, these authors showed that a compensatory rise in glycolysis and increased utilisation of glucose, to generate sufficient ATP, was observed. The effect was dose-dependent, with the highest levels usually causing little or no development. However, lower levels allowed relatively

normal levels of development to occur. Therefore, partial inhibition of oxidative metabolism was not detrimental to these embryos. More recently, Donnay and Leese (1999) have shown that re-expansion of the blastocoel cavity following oubain-induced collapse in cattle blastocysts, is largely dependent on oxidative phosphorylation. However, partial inhibition of oxidative phosphorylation with cyanide (or uncoupling with 2,4-dinitrophenol) did not prevent re-expansion, demonstrating some tolerance to inhibition of oxidative phosphorylation in maintenance of the blastocoel cavity (Donnay and Leese, 1999).

Summary of the Invention

It has now surprisingly been found that a partial reduction in atmospheric oxygen tension or partial inhibition of oxidative phosphorylation by the addition of an inhibitor or uncoupler, both timed to occur during the compaction process, improves the developmental ability and quality of pre-implantation embryos during produced *in vitro* culture.

It is therefore an object of the present invention to develop an *in vitro* embryo culture system that improves the development ability and quality of the embryos over those produced by other known culture systems. It is also an object of the present invention to provide compositions for use in such a system and pre-implantation embryos produced by such a system. It is also an object of the invention to provide a kit for carrying out the *in vitro* embryo culture system of the present invention or at least to provide the public with a useful choice.

According to the present invention there is provided a method of improving the developmental ability and quality of *in vitro* cultured pre-implantation embryos comprising the steps:

a) Culturing pre-implantation embryos *in vitro* to the pre-compaction or pre-blastulation stage;

b) Reducing the capacity of the embryos to generate ATP by oxidative phosphorylation either by partially reducing the oxygen content of the culture atmosphere or by the use of one or more selective oxidative phosphorylation inhibitors and/or uncouplers;

c) Culturing the embryos to the compaction or blastocyst stage; and

d) Assessing the developmental ability and quality of the embryos for suitability for implantation.

The embryos of step a) may be 1- or 2- cell early stage embryos. The embryos of step a) are preferably cultured for 3 to 4 days to reach pre-compaction and/or pre-blastulation stage in known culture media under a humidified atmosphere comprising 5 to 7% CO_2, 5 to 7% oxygen and the balance made up of nitrogen. The oxygen of the atmosphere of the cultured embryos may be varied at step b) from 0% to 7%, with the difference in oxygen content being replaced by nitrogen.

The method of the present invention may also be useful for improving the viability of cells in culture which have been derived from embryos, such as embryonic stem cells, or for culturing the embryos as a source of embryonic cells for nuclear transfer or for embryonic stem cell production.

Preferably, the energy substrates are carbohydrates, carboxylic acids and amino acids.

The one or more oxidative phosphorylation inhibitors may be selected from the group of known oxidative phosphorylation inhibitors, eg sodium azide (NaN_3), cyanide, rotenone and antimycin A, or from any other group of compounds which act as inhibitors of oxidative phosphorylation.

The one or more oxidative phosphorylation uncouplers may be selected from the group of known oxidative phosphorylation uncouplers, eg 2,4-dinitrophenol (DNP) and carbonylcyanide p-trifluoro-methyoxyphenylhydrazone (FCCP) or from any other group of compounds which act as uncouplers of oxidative phosphorylation from electron transport.

The oxidative phosphorylation inhibitor and/or uncoupler may be present in the culture media at a concentration of from 0.001 to 1000μM, preferably from 1.0 to 250μM, more preferably from 1.0 - 100 μM and most preferably 1.0 to 20μM.

The Day 4 to 5 (3 to 4 days of culture) embryos of step b) are cultured at step c) for 2 to 3 days to reach the compaction or blastocyst stage and assessed at step d) at Day 7 of development.

The embryos produced by the method of the present invention may then be transferred to a recipient female mammal or may be maintained in *in vitro* culture while retaining viability for use in embryo transfer, IVF and/or genetic manipulation, or may be stored or frozen prior to embryo transfer or other manipulation. In addition, the embryos produced by this method may be used as a source of embryonic cells for nuclear transfer or for embryonic stem cell production.

The pre-implantation embryos may be selected from the group of mammals consisting of cows, pigs, sheep, humans, horses, goats and other domestic animals. Preferably the pre-implantation embryos are cattle embryos.

The pre-implantation embryos may have been obtained from superovulation and standard collection procedures in fertilised mammals, from *in vitro* fertilisation procedures or *in vitro* nuclear transfer (of either somatic cells or embryonic or embryonic-derived cells) procedures and may have been pre-frozen.

According to a second aspect, the present invention provides a culture medium for improving the developmental ability and quality of pre-implantation embryos comprising at least one oxidative phosphorylation inhibitor and/or oxidative phosphorylation uncoupler wherein the inhibitor is selected from the group consisting of sodium azide, cyanide, rotenone and antimycin A, and any other group of compounds which act as inhibitors of oxidative phosphorylation, and the oxidative phosphorylation uncoupler is selected from the group consisting of 2,4-dinitrophenol

(DNP), carbonylcyanide p-trifluoro-methoxyphenylhydrazone (FCCP) or any other group of compounds which act as uncouplers of oxidative phosphorylation from electron transport in combination with an animal (eg mammalian) embryo maintaining medium.

The oxidative phosphorylation inhibitor and/or uncoupler may be present in the culture medium at a concentration of from 0.001 to 1000μM, preferably from 1.0 to 250μM, more preferably from 1.0 - 100μM and most preferably from 1.0 to 20μM.

The animal embryo maintaining medium may comprise a commercially available medium, and it is to this medium to which is added the oxidative phosphorylation inhibitors and/or uncouplers of the present invention. The commercially available medium may comprise a medium for culturing, storing, manipulating and/or freezing pre-implantation embryos. The culture medium of the invention is expected to allow for better preservation of frozen and stored embryos and to maintain and/or enhance the developmental ability and quality of these embryos in culture.

The present invention also provides a kit for improving the developmental ability and quality of *in vitro* cultured pre-implantation embryos comprising:

a) commercially available medium suitable for culturing pre-implantation embryos to blastulation and/or compaction;
b) one or more oxidative phosphorylation inhibitors and/or uncouplers in an amount sufficient to enhance the developmental ability and quality of the pre-implantation embryos;

wherein the one or more oxidative phosphorylation inhibitors and/or uncouplers is added to the medium when embryos are at the pre-compaction and pre-blastulation stage.

The one or more oxidative phosphorylation inhibitors are selected from the group consisting of known inhibitors, eg, sodium azide, cyanide, rotenone and antimycin A and from any other group of compounds which act as an inhibitor of oxidative phosphorylation.

The one or more oxidative phosphorylation uncouplers are selected from the group consisting of 2,4-dinitrophenol (DNP), carbonylcyanide p-trifluoro-methoxyphenylhydrazone (FCCP) or any other group of compounds which act as uncouplers of oxidative phosphorylation from electron transport.

The amount of the one or more oxidative phosphorylation inhibitors and/or uncouplers added to the culture medium is sufficient to give a concentration of oxidative phosphorylation inhibitor and/or uncoupler in the culture medium of from 0.001 to 1000μM, preferably 1.0 to 250μM, more preferably 1.0-100μM and most preferably 1-20μM.

The present invention also provides pre-implantation embryos produced by the method of the invention. The pre-implantation embryos are selected from the group of mammals consisting of cows, pigs, sheep, humans, horses, goats and other domestic animals.

Description of the Figures

The invention will be further described by reference to the following examples and also to the drawings in which :

Figure 1 shows the effect of oxygen concentration from Day 5 to 7 of development on cleaved embryo development to the compact morula and blastocyst stage (closed circles), Grade 1 and 2 compact morula and blastocyst stage (open circles) and Grade 1 and 2 blastocyst stage (closed triangles) during incubation in SOFaaBSA medium (Experiment 1):

Figure 2 shows development of cleaved bovine embryos to the compact morula and blastocyst stage (closed circles) and Grade 1 and 2 compact morula and blastocyst stage (open circles) when incubated in SOFaaBSA medium supplemented with NaN_3 (0, 5, 10 and 20 μM) from Day 5 to Day 7 of development (Experiment 2b). Different letters indicate significant differences ($P<0.05$); and

Figure 3 shows development of cleaved bovine embryos to the compact morula and blastocyst stage (closed circles) and Grade 1 and 2 compact morula and blastocyst stage (open circles) when incubated in late medium supplemented with antimycin A (0, 1, 2 and 5 μM) from Day 5 to Day 7 of development (Experiment 4).

DETAILED DESCRIPTION OF THE INVENTION

Definitions

By "developmental ability" is meant the ability of a zygote and/or embryo to develop through the morphologically identifiable stages of development up to a chosen developmental end point including the birth of a live born offspring.

By "embryo quality" is meant the developing embryo has morphologically and/or biochemical characteristics which can be used to grade the embryo for the purpose of prospectively measuring the embryo's developmental ability (Bavister, 1995; Barnett and Bavister 1996).

Description of the Preferred Methods and Embodiments

1. Production of embryos

Ovaries were collected from an abattoir and transported to the laboratory in saline (approximately 35 °C), the maximum time from slaughter to oocyte collection was approximately 3 h. Cumulus-oocyte complexes (COC) were recovered by aspiration of

1-5 mm follicles using an 18 g needle under vacuum (50mm Hg). The COC's were collected into Hepes-buffered TCM 199 medium (with Earle's salts, Life Technologies, N.Z.) supplemented with 10 μg/ml heparin (from porcine intestinal mucosa, Sigma, USA) and 0.4% w/v bovine serum albumin (BSA, affinity column purified, "ABRD", Immuno-Chemical Products, NZ). Prior to *in vitro* maturation, COC's were assessed morphologically and only those that had a compact, non-atretic cumulus oophorus-corona radiata and an oocyte with homogeneous cytoplasm were selected. All selected COC's were washed thoroughly in Hepes-buffered TCM 199 medium supplemented with 10% v/v fetal calf serum (FCS, Life Technologies), washed once in maturation medium and placed in 50 μl drops (10 per drop) of the same medium under oil and incubated for 24 h at 39°C under humidified 5% CO_2 in air. The medium used for maturation was TCM 199 supplemented with 10% FCS, 10μg/ml ovine follicle stimulating hormone ("Ovagen", Immuno-Chemical Products), 1 μg/ml ovine luteinizing hormone (Immuno-Chemical Products), 1 μg/ml oestradiol (Sigma) and 100 μM cysteamine (Sigma).

Spermatozoa were prepared from frozen-thawed semen, obtained from a sire which had been previously characterised as suitable for *in vitro* fertilisation in our laboratory. The contents of two 0.25 ml straws (each containing approximately 1 x 10^8 sperm/ml) were layered upon a Percoll gradient (45%/90%) and motile sperm were collected following centrifugation at approximately 1200 g for 20 min at room temperature. The motile fraction was washed once in Hepes-buffered Tyrode's Albumin Lactate Pyruvate medium (TALP) and then re-suspended to a final concentration at insemination of 2 x 10^6 sperm/ml in fertilisation medium, a modified TALP (Lu *et al.*, 1987), supplemented with 20 μM penicillamine (Sigma),10 μM hypotaurine (Sigma) and 10 μg/ml heparin (Sigma). Insemination was performed in 50 μl of fertilisation medium in microdrops under oil (approximately 5 oocytes per drop) over a 24 h period under the same conditions as described for oocyte maturation.

A description of the formulations and media referred to is attached as Annex I.

2. Statistical analysis

Unfertilised oocytes were excluded from all analyses. The proportional data for *in vitro* development of embryos were analysed, following logit transformation, using the Generalised Linear Models (GLM) procedure within the Genstat statistical package, or in the case of Experiment 7, metabolic data was compared by analysis of variance, followed by Tukey's test for multiple comparisons of individual means. In addition to the main treatment groups, the data were also tested for random effects of day of oocyte collection. Cell numbers and metabolic data were handled in a similar fashion, with the difference that transformation of the data using logs was only conducted when heterogeneity of variance was observed. Statistical significance was defined as $P \leq 0.05$.

Experiment 1 - Effect of Oxygen concentration

Following insemination, putative zygotes were removed and washed twice in a Hepes-buffered version of Synthetic Oviduct Fluid (SOF) medium (Tervit *et al.*, 1972), comprising of 20 mM Hepes, 5 mM $NaHCO_3$ and 3 mg/ml BSA (fatty acid-free, Sigma) (H-SOF) and were usually placed in 20 µl micro-drops of the modified SOF medium, SOFaaBSA, which includes MEM essential and non-essential amino acids (Life Technologies) and 8mg/ml BSA (Gardner *et al.*, 1994) and incubated under humidified 5% CO_2, 7%O_2 and 88% N_2 at 38.5°C. Embryos were transferred to fresh media on Day 5 of development (Day 0 = day of insemination) and randomly allocated to one of five 5% CO_2 atmospheres which differed in the content of oxygen (and nitrogen, used to balance the atmospheres). The five atmospheres were: 0%, 1%, 2%, 4% and 7% O_2. All embryos were removed from culture on Day 7 of development and stage of development (and quality of those that reached compacted morulae or blastocyst stages) was recorded after examination under a dissecting microscope. These experiments were conducted over 5 replicates using a total of 871 cleaved embryos. Grade 1 and 2 blastocyst stage embryos were removed for cell number analysis. Cell numbers were determined using fluorescence microscopy and image

analysis (Video Pro, Adelaide, Australia), following fixation in acetic acid:ethanol:H_2O (3:2:1) and staining with propridium iodide (1% w/v).

The results for embryo development are described in Figure 1. A quadratic trend ($p<0.1$) was observed for development to the compacted morulae and blastocyst stage and for the development of Grade 1 and 2 embryos, indicating that development was improved with O_2 levels below 7% but above 0%. However, significance was only observed for the proportion of Grade 1 and 2 blastocysts ($P<0.05$) (Figure 1). The cell number within blastocysts adjudged Grade 1 and 2 quality, derived from different oxygen concentrations, did not vary and contained on average 118±5.0 cells/embryo.

Experiment 2 –Addition of an oxidative phopshorylation inhibitor, NaN_3

Embryos were produced and treated similarly as in Experiment 1, but at Day 5 of development, embryos were transferred to fresh medium containing one of 4 levels of NaN_3 (0, 10, 100 and 1000 µM). Culture was performed within 4-well plates, each well containing one of the 4 concentrations (20 µl medium). Each microdrop was overlaid with 0.5 ml mineral oil. Embryos were cultured under humidified 5%CO_2, 7% O_2 and 88% N_2 at 38.5°C. As with Experiment 1, culture ended on Day 7 and development was recorded after examination under a dissecting microscope. Within this experiment 3 replicates were conducted using a total of 335 cleaved embryos. On the basis of the first experiment (i.e. Experiment 2a), a further dose-response trial was initiated. Levels of NaN_3 assessed were 0, 5, 10 and 20 µM, over 3 replicates using 342 cleaved embryos (Experiment 2b).

The results of Experiment 2a are presented in Table 1. Significant (log linear) differences were observed between the three NaN_3-supplemented solutions for the proportion of embryos developing to compacted morulae and blastocysts (% CM + BL, $P<0.001$), Grade 1 and 2 (G 1 & 2) CM + BL ($P<0.001$). In all cases the highest level of development was achieved in 10 µM NaN_3 and lowest in 1000 µM. No difference was observed for development of embryos between the response obtained for the 0

(control) and 10 μM NaN_3 treatments.

Table 1. Development of bovine embryos when incubated in SOFaaBSA medium supplemented with NaN_3 from Day 5 to Day 7 of development (Experiment 2a).

NaN_3 (μM)	% CM + BL [1,3]	% Grade 1 & 2 [2,3]
0 (control)	40±5.4[a]	26±4.8[a]
10	46±5.3[a]	37±5.1[a]
100	34±5.3[a]	13±3.7[b]
1000	5±2.4[b]	1±1.2[c]

[1] Proportion of compact morula and blastocyst stage embryos from cleaved embryos.

[2] Proportion of Grade 1 and 2 (transferable quality) compact morula and blastocyst stage embryos from cleaved embryos

[3] Differing superscripts differ significantly (a,b,c $P<0.05$)

The results for Experiment 2b are shown below in Figure 2. In contrast to the previous experiment, the average NaN_3 response was significantly higher than the control embryos for all levels of embryo development ($P<0.01$). Furthermore, a significant linear decrease ($P<0.01$) with increasing NaN_3 concentration was observed for % G1 & 2 CM + BL.

In addition to the developmental data, visual assessment of the inhibitor treated embryos provided evidence that they were also of superior quality to control embryos, in that the inner cell mass of these embryos appears, visually at least, to be larger and "denser".

Experiment 3 - Addition of NaN_3 from Day 1 of development.

Embryos were produced using the same basic conditions described in Experiments 1 and 2, with some minor differences. Firstly, the culture system was a sequential system (e.g. Gardner, 1998), based on the principles outlined elsewhere (Thompson, 1996). Using this media system, embryos are cultured in an early development medium (early medium) for 4 d and then transferred to a peri-compaction and blastulation medium (late medium) for a further 2 d. Secondly, this experiment was designed to assess the effect of NaN_3 from Day 1 of development (first day of culture). Putative zygotes were cultured in early development medium in either 0, 10 or 100 μM NaN_3 for 4 days and then transferred into fresh drops of late medium. Culture conditions and morphological observations were conducted as previously described. Four replicates were performed using a total of 602 cleaved embryos.

The results of this experiment are presented in Table 2. Addition of 10 μM NaN_3 on Day 1 of embryo development, i.e. the start of embryo culture, inhibited development significantly ($P<0.001$). Furthermore, 100 μM was found to be highly toxic.

Table 2. Development of bovine embryos when incubated in late medium supplemented with NaN_3 from Day 1 to Day 5 of development (Experiment 3).

NaN_3 (μM)	% CM + BL[1]	% Grade 1 & 2[2]
0	54±2.8	41±2.8
10	39±2.7	26±2.5
100	0	0
P	<0.001	<0.001

[1] Proportion of compact morula and blastocyst stage embryos from cleaved embryos.
[2] Proportion of Grade 1 and 2 (transferable quality) compact morula and blastocyst stage embryos from cleaved embryos

Experiment 4 - Addition of Antimycin A

Embryos were produced using the same conditions as described in Experiment 3. This experiment examined the effect of antimycin A, another oxidative phosphorylation inhibitor (acts at the level of cytochrome oxidase b). Levels assessed were 0, 1, 2, and 5 μM, over 5 replicates (N = 1040 cleaved embryos).

Results of this experiment are presented in Figure 3. Antimycin A addition at Day 5 of development was found to significantly inhibit further development ($P<0.001$), even at levels as low as 1 μM.

Experiment 5 - Metabolism of embryos following treatment with metabolic inhibitor

Embryos were produced using conditions already described in Experiment 3. The aim of this experiment was to determine if the resulting transferable quality embryos derived from culture in medium containing different levels (0-100 μM) of NaN_3 also differed in their metabolic profile. At the cessation of culture, embryos were morphologically graded and transferred to a modified late medium, the modification being the replacement of 25 mM $NaHCO_3$ with 20 mM Hepes and 5 mM $NaHCO_3$ (the latter medium is referred to as Transfer medium). In addition, Transfer medium also included the corresponding level of NaN_3 used for the culture of embryos. Metabolic profile of embryos was characterised by measuring the consumption of oxygen, glucose and pyruvate, and the production of lactate, performed using techniques described elsewhere (Houghton *et al.*, 1996, Thompson *et al.*, 1996). Following metabolic assays, cell numbers were determined as described above. Five replicates were conducted, using a total of 1192 cleaved embryos.

The developmental and cell number data associated with the production of embryos cultured in NaN_3 is described in Table 3. There were significant ($P<0.001$) linear decreases (on a logarithmic scale) for both % CM + BL and % Grade 1 and 2 between the two levels of NaN_3 used. However, only for the proportion of Grade 1 and 2

embryo development was there evidence of an optimal dose ($P<0.05$, i.e. a quadratic function). There was no significant difference in development rates between 0 (control) and 10 μM NaN_3. However, cell numbers significantly differed between the three levels used, with 10 μM NaN_3 yielding expanded blastocyst stage embryos with the highest numbers of cells (Table 3).

Table 3. Development and cell numbers of bovine embryos when incubated in late medium supplemented with NaN_3 from Day 5 to Day 7 of development (Experiment 5).

NaN_3 (μM)	% CM + BL[1,3]	% G1 & 2[2,3]	Cell number[4] (Blastocyts)
0 (control)	57±3.0[a]	45±3.0[a]	103±5.2[a]
10	60±2.9[a]	50±2.9[a]	119±5.2[b]
100	24±1.8[b]	13±1.4[b]	73±4.5[c]

[1] Proportion of compact morula and blastocyst stage embryos from cleaved embryos.

[2] Proportion of Grade 1 and 2 (transferable quality) compact morula and blastocyst stage embryos from cleaved embryos

[3] Differing superscripts differ significantly (a,b $P<0.001$)

[4] Differing superscripts differ significantly (a,b $P<0.05$, a,c $P<0.001$, b,c $P<0.001$)

The effect of incubation in NaN_3 on embryo metabolism is presented in Table 4. No real difference was observed between 0 and 10 μM NaN_3 levels, except a log linear increase in glucose consumption was observed ($P<0.05$). A similar increase in lactate production was also observed but this was variable and not significant. Only at 100 μM NaN_3 was a significant reduction ($P<0.05$) in oxygen uptake observed.

Table 4. Effect of NaN_3 in the culture medium on metabolism of bovine blastocyst stage embryos (Experiment 5).

NaN_3 (μM)	O_2 uptake (nl/embryo/h)	Pyruvate uptake (pmol/embryo/h)	Glucose uptake (pmol/embryo/h)	Lactate prodn. (pmol/embryo/h)
0 (control)	1.1±0.20[a]	5.2±0.54	12±3.2[a]	22±6.0
10	1.1±0.19[a]	5.2±0.51	20±3.1[b]	28±5.7
100	0.4±0.11[b]	5.4±0.69	29±4.5[c]	38±7.7

Differing superscripts differ significantly ($P<0.05$)

Experiment 6 - Examination of embryo development following treatment with a metabolic uncoupler, 2,4-dinitrophenol.

A further experiment was conducted to further examine the effect of the addition of DNP (0-1000 μM) on Day 5 of development. Embryos were produced using the techniques described in Experiment 3. A total of 10 replicates were conducted (N = 2233), with embryos produced in the last 5 replicates used for metabolic determinations. Following metabolic assays, cell numbers were determined using techniques described above.

The development of bovine embryos and the resulting cell numbers of expanded blastocyst stage embryos when incubated in DNP (0-1000 μM) is described in Table 5. A highly significant ($P<0.001$) log quadratic function was observed for total embryo development and development of Grade 1 and 2 embryos. Optimal development appears to lie between 10 and 100 μM DNP. Cell number increased significantly in the presence of 10 μM and 100 μM DNP compared to its absence.

Table 5. Effect of addition of DNP on Day 5 of development on subsequent development to Day 7 of bovine IVP embryos (Experiment 6).

2, 4-dinitrophenol (μM)	% CM + BL[1,3]	% G1 & 2[2,3]	Cell number[4] (Expanded blastocyst)
0 (control)	51±2.0[a]	40±1.9[a]	121±3.7[a]
10	60±1.9[b]	50±1.9[b]	141±4.9[b]
100	55±2.3[a,b]	43±2.3[a]	140±4.4[b]
1000*	6±1.4[c]	2±0.7[c]	-

*Only the first five replicates used 1000 μM, as it was found to be highly toxic.

[1] Proportion of compact morula and blastocyst stage embryos from cleaved embryos.

[2] Proportion of Grade 1 and 2 (transferable quality) compact morula and blastocyst stage embryos from cleaved embryos

[3]Differing superscripts differ significantly (a,b $P<0.05$, a,c $P<0.001$, b,c $P<0.001$)

[4]Differing superscripts differ significantly (a,b $P<0.001$)

Experiment 7 - The effect of 10 μM 2,4-dinitophenol on the metabolism of glucose and pyruvate by day-5, -6 and -7 bovine in vitro produced embryos.

The objective of this experiment was to determine whether metabolic effects of 10 μM DNP could be detected in day-5 to day-7 cattle embryos using a technique that measures the production of $^{14}CO_2$ and $^{3}H_2O$ from radiolabelled substrates. This technique provides more specific information about energy metabolism in early embryos than does uptake, and has been used successfully for studies of the changes in metabolic activity during early development (e.g. Rieger *et al.*, 1992). The production of $^{14}CO_2$ from [2-^{14}C]-pyruvate was used a measure of oxidative metabolism and the production of $^{3}H_2O$ from [5-^{3}H]-glucose was used a measure of anaerobic glycolysis. Embryo production was the same as described for Experiment 6. On Day 5, the

embryos (compacted morula stage) in each trial were divided into six equivalent groups; three groups were transferred to late medium containing 10 μM DNP (DNP treatment), and three groups were transferred in to late medium without DNP (Control treatment). One of each of the DNP and control groups were taken immediately for measurement of metabolic activity, as described below. The remaining four groups were placed in culture in 20 μl drops under oil under 5% CO_2, 7% O_2, 88% N_2, at 38.5°C. On Day 6 (early blastocyst stage) and on Day 7 (late to expanded blastocyst stage), one of each of the DNP and control groups were taken for measurement of metabolic activity.

The measurement of metabolic activity was done as described by Rieger *et al.* (1992). Briefly, the DNP and control embryos were transferred to 800 μl volumes of late medium containing 10 μM DNP, and late medium without DNP, respectively, and cultured under 5% CO_2 in air, at 38.5 °C for approximately 90 min. Just before being placed in the metabolic measurement apparatus, the control embryos were transferred to a final 800 μl wash of late medium without DNP, and the DNP embryos were transferred to a final 800 μl wash of late medium containing 20 μM DNP. The embryos were then taken up individually in 2 μl of the final wash and placed in the cap of a 2 ml cryotube. To this was added 2 μl of late medium without DNP containing a mixture of [2-^{14}C]-pyruvate and [5-^{3}H]-glucose. The final total concentrations (unlabelled plus labelled) of pyruvate and glucose were 0.96 ± 0.01 mM (mean ± s.e.m.), and 1.5 mM, respectively. The caps were then placed onto the top of the cryotubes, which had been loaded with 1.5 ml of 25mM $NaHCO_3$ that had been saturated with 5% CO_2, 7% O_2, 88% N_2. The bicarbonate serves as a trap for the $^{14}CO_2$ and $^{3}H_2O$ produced by metabolism of the radiolabelled substrates. The tubes were then cultured at 38.5 °C for 3 h.

At the end of the metabolic measurement period, the caps were removed and the bicarbonate contents of each tube transferred to a 20 ml scintillation vial containing

200 µl of 0.1 N NaOH that serves to convert the bicarbonate and dissolved CO_2 to carbonate. To this was added 15 ml of scintillation fluid, and the vials were counted for 5 min each in a counter programmed for automatic dual-label correction to disintegrations per minute (d.p.m.). Three sham preparations containing everything except an embryo were included with each treatment of each assay to correct for chemiluminescence and non-specific transfer of radioactivity to the bicarbonate solution. The amount of each substrate metabolized by each embryo was calculated by subtracting the mean sham d.p.m. from the d.p.m. for the embryo, dividing by the total d.p.m. in the metabolic measurement droplet, and multiplying by the total amount of substrate (unlabelled plus labelled).

The embryos were recovered from the metabolic measurement droplets immediately following the measurement period, transferred as a group to 800 µl volumes of late medium containing 10 µM DNP (DNP group), or late medium without DNP (Control group) and cultured under 5% CO_2 in air at 38.5 ^{o}C. The morphological development of all embryos was assessed on Day 8.

Of the embryos subjected to the metabolic measurement, 27/42 (64.3%) of the day-5 embryos, 28/43 (66.7%) of the day-6 embryos, and 31/33 (93.9%) of the day-7 embryos developed to the expanded or hatched blastocyst stage by Day 8. These are considered to be within acceptable norms and therefore the metabolic measurement data are considered to be reliable.

The effects of treatment and day on the metabolism of pyruvate are shown in Table 6. There were highly significant ($P \leq 0.001$) overall effects of treatment and day, but no significant effect of interaction. The metabolism of pyruvate was significantly greater in the DNP group than in the control group on each of Days 5, 6, and 7. Within both the control and DNP groups, there were significant effects of day, such that pyruvate metabolism increased significantly on each subsequent day.

Table 6. The effect of DNP on the mean (± s.e.m.) metabolism of pyruvate by day-5, -6, and -7 bovine embryos.

	Control		DNP	
Day	No. of embryos	pmols/ embryo/3 h	No. of embryos	pmols/ embryo/3 h
5	21	3.29 ± 0.28^{a}	21	$4.25 \pm 0.31^{a*}$
6	21	4.78 ± 0.35^{b}	21	$6.14 \pm 0.43^{b*}$
7	14	9.86 ± 0.97^{c}	19	$13.28 \pm 0.61^{c*}$

^{abc}Within columns, means with no common superscripts are significantly different ($P \leq 0.05$)

*Significantly different from the mean for the control group on the same day. ($P \leq 0.05$)

The effects of treatment and day on the metabolism of glucose are shown in Table 7. There were highly significant ($P \leq 0.01$) overall effects of treatment and day, but no significant effect of interaction. The metabolism of glucose was not different between the DNP group and the control group on Days 5 or 6, but was significantly greater in the DNP group than in the control group on Day 7. Within the control group, there was a significant effect of day, and glucose metabolism increased significantly from Day 5 to Day 6, but was not significantly different between Days 6 and 7. Within the DNP group there was a significant effect of day, such that glucose metabolism increased significantly on each subsequent day.

Table 7. The effect of DNP on the mean (± s.e.m.) metabolism of glucose by day-5, -6, and -7 bovine embryos.

	Control		DNP	
Day	No. of embryos	pmols/ embryo/3 h	No. of embryos	pmols/ embryo/3 h
5	21	9.77 ± 0.83^{a}	21	11.29 ± 1.05^{a}
6	21	17.27 ± 1.45^{b}	21	18.31 ± 1.41^{b}
7	14	23.08 ± 2.45^{b}	19	$33.26 \pm 2.49^{c*}$

^{abc}Within columns, means with no common superscripts are significantly different ($P \leq 0.05$)

*Significantly different from the mean for the control group on the same day. ($P \leq 0.05$)

The results of this experiment clearly demonstrate that 10 μM DNP significantly increases the metabolism of pyruvate in cattle embryos from Day 5 to Day 7, over the entire period when it is routinely included in late medium. The metabolism of glucose in significantly increased only on Day 7, which probably reflects the increased energy demands associated with blastocyst expansion and hatching.

Experiment 8 - Post-transfer survival of embryos following treatment with 10 μM DNP.

Embryos were produced using conditions described above in Experiment 6. On Day 5 of development, embryos were transferred into late medium supplemented with 10 μM DNP. On Day 7 of development, morphology was determined and embryos of Grade 1

and 2 quality (i.e. transferable quality) were allocated to be transferred to suitably synchronised recipient parous cows (N = 24). All cows received an embryo ipsilateral to the functional corpus luteum. A further group of synchronised recipients (N = 45) were artifically inseminated using frozen semen from the same bull used to produce embryos *in vitro*. On Day 35 of pregnancy, all cows were scanned by ultrasonography to determine pregnancy status. Of the 24 cows receiving a single Grade 1 or 2 quality embryo by a standard non-surgical transfer technique, 12 (50%) were pregnant following ultrasonography. This compares favourably with 49% (22/45) of cows scanned pregnant following artificial insemination.

Experiment 9 – Effect of NaN_3 on pig embryo development in vitro.

The aim of this experiment was to determine if NaN_3 (0, 5, 10, or 20 μM) had similar effects on development during the peri-compaction period in pig embryos as had been observed for cattle embryos.

Pig oocytes were matured for 42 to 44 h in a defined protein-free medium and co-incubated with frozen-thawed boar spermatozoa as reported previously (Prather and Day 1998). Presumptive zygotes were then cultured in NCSU 23 medium with 0.4% BSA for 4 days. At that time embryos that reached the compact morula stage were selected (n=462) and transferred to culture medium containing 0, 5, 10, or 20 μM sodium azide (NaN_3), an inhibitor of oxidative phosphorylation. Following an additional 2-day culture, embryonic development was recorded and the embryos were subsequently stained with Hoechst 33342 to assess the number of nuclei. To evaluate developmental potential, 14 treated embryos were transferred surgically into a ligated uterine horn of a recipient pig; 12 non-treated blastocysts were transferred into the contralateral horn. Twenty-five days later the number of fetuses that developed on either side was determined following hysterectomy.

The inhibition of oxidative phosphorylation at the time of compaction had no effect on the rate of blastocyst formation, which ranged between 72.1 and 79.7 %. However,

NaN_3 treatment of the compact morulae resulted in increased number of nuclei in the developing embryos. The average number of nuclei in the control embryos was 18.7±0.8, and this was significantly lower ($p<0.05$) than that in the groups where oocytes were treated with 10 or 20 μM NaN_3 (22.6±1.1 and 23.3±0.8, respectively). The result of embryo transfer showed that these embryos were viable: 4 of 14 NaN_3-treated embryos developed into fetuses, while of the 12 control embryos, 1 fetus was recovered. Therefore, as for cattle embryos, transient inhibition of oxidative phophorylation, induced by NaN_3 during the peri-compaction period has a positive effect on the developmental quality of pig embryos produced in vitro.

3. Discussion

The azide ion (N_3^-) inhibits oxidative phosphorylation by inhibiting the electron transport cascade, specifically by inhibiting cytochrome oxidase a_3, as does cyanide. Therefore, both electron transport and oxidative phosphorylation are down-regulated. There are several other inhibitors in this class, including rotenone and antimycin A, and are distinct from those which inhibit oxidative phosphorylation itself, such as oligomycin. Uncouplers, such as 2, 4-dinitrophenol, do not inhibit these pathways, but separate the two. Therefore in the presence of uncouplers, electron transport can occur without concomitant ATP synthesis, a result which usually causes an increase in TCA-cycle activity and oxygen consumption (Newsholme and Leech, 1983). 2, 4-dinitrophenol (DNP) has been used previously by us to demonstrate that, normally in ovine blastocysts, little glucose is oxidised by the TCA-cycle (Thompson *et al.*, 1991).

The data described here provide evidence that partial (sub-acute) down-regulation of mitochondrial ATP production during the compaction and blastulation stages of cattle and pig in *vitro* produced embryos, improves *in vitro* development. This was demonstrated three ways: 1) by physiologically decreasing oxygen availability; 2) by partially inhibiting electron transport using NaN_3; 3) by partially uncoupling oxidative phosphorylation from electron transport, using DNP. Nevertheless, as also demonstrated by Donnay and Leese (1999), we have shown that ATP production via.

oxidative phosphorylation is an essential pathway for cattle embryo development at all stages of development. The novelty of our observations is that partial inhibition during the peri-compaction period is beneficial to development in at least 2 unrelated species (cattle and pig). This is not the same for rodent embryos. Brison and Leese (1994) demonstrated that inhibition of oxidative phosphorylation (using as high as 1 mM cyanide) in rat morula stage embryos had little effect on blastulation rates, but these authors did not describe an increased developmental capacity. In contrast, 1 mM cyanide or DNP is completely inhibitory to mouse embryo development when administered at either the 2-cell or blastocyst stage (Thomson 1967). Development was only partially retarded at levels of these compounds of approximately 10 to 100 μM, especially when included at Day 3 of development (Thomson 1967).

The mechanism for the beneficial effect described here is unclear, but most likely involves the relative contribution of glycolytic ATP vs. mitochondrial ATP production and could involve the establishment of an appropriate redox state which encourages increased glucose metabolism. Maintenance of an appropriate redox state was also suggested by us as a mechanism for the enhanced development of ovine embryos in vitro under [pyruvate]:[L-lactate] ratios of 1:5 (Thompson *et al.*, 1993) and later supported by observations of Edwards *et al.* (1997).

The effectiveness of inhibitors and uncouplers varied. Sodium azide appears to be beneficial over a narrow range of concentrations (5-20 μM). There was little effect on oxygen uptake with 10 μM, but there was an increase in glucose consumption without a significant corresponding increase in lactate production, which supports our suggestion that perhaps partial inhibition of mitochondrial ATP production creates a more favourable redox state, and this in turn increases the availability of biosynthetic intermediates, potentially through the pentose phosphate and/or Embden-Meyerhoff (glycolytic) pathway. Encouragingly, an increase in glucose consumption has been shown to be correlated to increased embryo viability in post-hatching stage bovine blastocysts (Renard *et al.*, 1980). At higher NaN_3 concentrations, there is sufficient down regulation of electron transport and O_2 consumption to inhibit further

development, although even at 100 μM, some embryos do still reach the blastocyst stage. This provides further evidence that mitochondrial ATP generation is still necessary for development to occur. Furthermore, the effect of NaN_3 was not always consistent between experiments. Although significantly effective at the 5-10 μM range in Experiment 2 (using SOFaaBSA medium), this was not observed in Experiment 5. We suspect that the effect of NaN_3 may be conditional on the time of year, possibly mediated through the developmental competence of oocytes collected, which may be related to the condition of animals at the time of slaughter.

Antimycin A was inhibitory at doses of 1 μM and higher, demonstrating its relative potency as an inhibitor of electron transport. As no stimulatory effect was observed, we did not investigate this compound further. Antimycin A inhibits at a different level of electron transport (cytochrome oxidase b) compared to NaN_3, and this could possibly explain the difference in response. However, at this stage we cannot rule out the possibility that an optimal dose may exist at a level below 1 μM.

The addition of DNP caused similar effects to the addition of NaN_3, in that an optimal dose was observed. Levels greater than 100 μM reduced embryo development, but as with NaN_3, a lower level (i.e. 10 μM) had a marked positive effect on both embryo development and cell number of resulting blastocysts. Examination of the metabolic profile of DNP-treated embryos revealed that, as expected, pyruvate (and to a lesser extent, O_2) utilisation increased with increasing concentrations of DNP. An increase in glucose metabolism was only observed at Day 7 of development, when blastocysts are expanding. Of significance was the high survival of embryos following transfer on Day 7 following incubation in DNP. This confirms that at such concentrations, these compounds appear not to be detrimental to subsequent survival.

The modulation of metabolic activity to improve embryo development has been observed in several other species. *In vitro* culture in the presence of physiological glucose levels has been shown to stimulate glycolytic behavior in a variety of cell types, including pre-implantation embryos (reviewed by Leese *et al.*, 1998). Such up-regulation of glycolysis (i.e. the "Crabtree effect") during early cleavage has been

shown to be detrimental to embryo development in several species (Bavister, 1995) and has been controlled by the lowering of glucose levels or its complete removal (e.g. mouse Chatot *et al.*, 1990; hamster Seshagiri and Bavister 1989; ovine Thompson *et al.*, 1992; bovine Rosenkrans *et al.*, 1993) or the addition of EDTA (100 μM) (Abramczak *et al.*, 1977). Gardner and Lane (1993) demonstrated that addition of EDTA inhibited glycolysis, most likely by chelating Mg^{2+} ions, which are an important co-factor for the activity of regulatory glycolytic enzymes. However, to our knowledge, the research described in this report is the first demonstration that post-compaction metabolism can be manipulated leading to improved development in any mammalian species. The significance of this lies in the observation that the time to first cleavage of bovine embryos in vitro has been found to correlate with successful development to the blastocyst stage (Holm *et al.*, 1998, Rieger *et al.*, 1999). However, here we have used common conditions for early cleavage and have randomly allocated all cleaved embryos to subsequent treatment groups on Day 5 of development. Thus the effect of these compounds is to "rescue" embryos that normally would not proceed.

Furthermore, application of this technology may also be useful in the culture and maintenance of cells derived from post-compaction embryos, in particular embryonic stem cells. Indeed, it has been reported that a reduction of oxygen concentration from 20% (atmospheric) to 7% facilitated growth of goat embryonic stem cell-like *cells in vitro* (Udy and Wells 1996).

INDUSTRIAL APPLICATION

The present invention provides methods and media useful in improving the developmental ability and quality of pre-implantation embryos for use in animal breeding programs to improve the overall success of *in vitro* fertilization techniques.

It will be appreciated that the present invention is not limited to the above examples only, many variations, such as might readily occur to a person skilled in the art being possible, without departing from the scope of the attached claims.

Appendix: Composition of a selection of media that have been used under defined or semi-defined conditions for cattle embryo culture. All values are expressed in mM units unless specified.

Component[a] (mM)	CDM (1)	CR1 (2)	CZB (3)	HECM (4)	SOFaaBSA(5)
NaCl	81	114.7	81.62	98.0	107.7
KCl	8	3.1	4.83	3.2	7.16
KH_2PO_4			1.18		1.19
$MgCl_2$	0.5			0.5	0.48
$MgSO_4$			1.18		
$NaHCO_3$	25	26.2	25.12	25.0	25.0
$CaCl_2$	2		1.7	2.0	1.71
Na pyruvate	0.5	0.4	0.27	0.5	0.33
Na lactate	5		31.3	10	3.3
Ca lactate		5			
Glucose	2				1.5
Glutamine	1	1	1	1	1.0
Essential amino acids	MEM[b]	BME[c]		Various amino acids	MEM
Non-essential amino acids	MEM + 5mM glycine	MEM		Various amino acids	MEM
EDTA	0.1		0.11		
BSA (mg/ml)		3	4		8
PVA (mg/ml)	0.1			1	

[a]Antibiotics are not included in the table, but are routinely used.
[b]Minimal Essential Medium, [c]Basal Medium Eagle's salts.

1. Seidel GE Jr, Glass T and Olson SE. Culture of 1-cell bovine embryos to blastocysts in chemically defined media. Biol. Reprod. 1991; 44 Suppl. 1: 155 (Abstr. 412).
2. Rozenkrans CF Jr and First NL. Effect of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes *in vitro*. J. Anim. Sci. 1994; 72: 434-437.
3. Chatot CL, Ziomek CA, Bavister BD, Lewis JL, and Torres I. An improved culture medium supports development of random-bred 1-cell mouse embryos *in vitro*. J. Reprod. Fertil. 1989; 86: 679-688.
4. Schini S and Bavister BD Two-cell block to development of cultured hamster embryos is caused by phosphate and glucose. Biol. Reprod. 1988; 39: 1183-1192.
5. Gardner DK, Lane M and Batt P. Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage *in vitro* in the absence of serum and somatic cells: Amino acids, vitamins, and culturing embryos in groups stimulate development. Biol. Reprod. 1994; 50: 390-400.

4. References

Abramczuk J, Solter D and Koprowski H (1977) The beneficial effect of EDTA on development of mouse one cell embryos in chemically defined medium. *Developmental Biology* **61** 378-383.

Bavister BD (1995) Culture of preimplantation embryos: facts and artifacts. *Human Reprouction Update* 1 91-148.

Brison DR and Leese HJ (1991) Energy metabolism in late preimplantation rat embryo. *Journal of Reproduction and Fertility* **93** 245-251.

Brison DR and Leese HJ (1994) Blastocoel cavity formation by pre-implantation rat embryos in the presence of cyanide and other inhibitors of oxidative phosphorylation. *Journal of Reproduction and Fertility* **101** 305-309.

Brown BW and Mattner PE (1984) Effects of ovarian hormones on ovarian capillary blood flow in anoestrous ewes. *Australian Journal of Biological Science* **37** 389-392.

Chatot CL, Lewis JL, Torres I and Ziomek CA (1990) Development of 1-cell embryo from different strains of mice in CZB medium. *Biology of Reproduction* **42** 432-440.

Donnay I and Leese HJ (1999) Embryo metabolism during the expansion of the bovine blastocyst. *Molecular Reproduction and Development* **53** 171-178.

Fischer B and Bavister BD (1993) Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. *Journal of Reproduction and Fertility* **99** 673-679.

Gardner DK (1998) Embryo development and culture techniques. In *Animal Breeding. Technology for the 21st Century* (ed. A. J. Clark), pp. 13-46. Harwood Academic Publishers, Amsterdam.

Gardner, DK and Lane M (1993) The 2-cell block in CF-1 mouse embryos is associated with an increase in glycolysis and a decrease in tricarboxylic acid (TCA) cycle activity: Alleviation of the 2-cell block is associated with the restoration of *in vivo* metabolic pathway activity. *Biology of Reproduction.* **49** *Supplement* **1** 52

Gardner DK, Lane M and Batt P (1993) Uptake and metabolism of pyruvate and glucose by individual sheep preattachment embryos developed *in vivo*. *Molecular Reproduction and Development* **36** 313-319.

Gardner DK, Lane MW and Lane M (1997) Bovine blastocyst cell number is increased by culture with EDTA for the first 72 hours of development from the zygote. *Theriogenology* **47** 278 (abstr).

Gott AL, Hardy K, Winston, RML and Leese HJ (1990) Non-invasive measurement of pyruvate and glucose uptake and lactate production by single human preimplantation embryos. *Human Reproduction* **5** 104-108.

Hewitson LC and Leese HJ (1993) Energy metabolism of the trophectoderm and inner cell mass of the mouse blastocyst. *Jouranl of Experimental Zoology* **267** 337-343.

Holm P, Shukri NN, Vatja G, Booth P, Bendixen C, and Callesen H (1998) Developmental kinetics of the first cell cycles of bovine in vitro produced embryos in relation to their in vitro viability and sex. *Theriogenology* **50** 1285-1299.

Johnson MH and Nasr-Esfahini MH (1994) Radical solutions to cultural problems: could free oxygen radicals be responsible for the impaired development of preimplantation mammalian embryos *in vitro*? *Bioessays* **16** 31-38.

Leese HJ (1991) Metabolism of the preimplantation mammalian embryo. *Oxford Reviews of Reproductive Biology* **13** 35-72.

Leese HJ, Donnay I and Thompson JG (1998) Human assisted conception: A cautionary tale. Lessons from domestic animals. *Human Reproduction* **13** (*Supplement* **4**) 101-119.

Newsholme EA and Leech AR (1983) *Biochemistry for the medical sciences*. John Wiley & Sons, Chichester, U.K.

Partridge RJ and Leese HJ (1996) Consumption of amino acids by bovine preimplantation embryos. *Reproduction, Fertility and Development* **8** 945-950.

Prather RS and Day BN (1998) Practical considerations for the in vitro production of pig embryos. *Theriogenology* **49** 23-32

Renard J-P, Philippon A and Menezo Y (1980) *In vitro* uptake of glucose by bovine blastocysts. *Journal of Reproduction and Fertility* **58** 161-164.

Rieger D and Guay P (1988) Measurement of the metabolism of energy substrates in individual bovine blastocysts. *Journal of Reproduction and Fertility* **83** 585-591.

Rieger D, Lonergan P and Boland M (1999) The relationship between the time of first cleavage of fertilized cattle oocytes and their development to the blastocyst stage. *Theriogenology* **51** 190 (abstr).

Rieger D, Loskutoff, NM and Betteridge KJ (1992) Developmentally related changes in the uptake and metabolism of glucose, glutamine and pyruvate by cattle embryos produced in-vitro. *Reproduction Fertility and Development* **4**: 547-557.

Rosenkrans CF, Zeng GQ, Mcmamara GT, Schoff PK and First NL (1993) Development of bovine embryos in vitro as affected by energy substrates. *Biology of Reproduction* **49** 459-462.

Seshagiri PB and Bavister BD (1989) Glucose inhibits development of hamster 8-cell embryos in-vitro. *Biology of Reproduction* **40** 599-606.

Thompson JG (1996) Defining the requirements for bovine embryo culture. *Theriogenology* **45** 27-40.

Thompson JG, Bell ACS, Pugh PA and Tervit HR (1993) Metabolism of pyruvate by pre-elongation sheep embryos and effect of pyruvate and lactate concentrations during in vitro culture. Reproduction, Fertility and Development **5** 417-423.

Thompson JG, Partridge, RJ, Houghton FD, Cox CI and Leese HJ (1996) Oxygen uptake and carbohydrate metabolism by *in vitro* derived bovine embryos. *Journal of Reproduction and Fertility* **106** 299-306

Thompson JG, Sherman ANM, Allen NW, McGowan LT and Tervit HR (1998) Protein content, synthesis and uptake in pre-elongation stage bovine embryos *Molecular Reproduction and Development* **50** 139-145

Thompson JG, Simpson AC, Pugh PA and Tervit HR (1992) Requirement for glucose during in vitro culture of sheep preimplantation embryos. *Molecular Reproduction and Development* **31** 253-257.

Thompson JGE, Simpson AC, Pugh PA Wright RW Jnr and Tervit HR (1991) Glucose utilization by sheep embryos derived *in vivo* or *in vitro*. *Reproduction, Fertility and Development* **3** 571-576.

Thomson JL (1967) Effects of inhibitors of carbohydrate metabolism on the development of preimplantation mouse embryo. *Exp. Cell Res.* **46** 252-262.

Udy GB and Wells DN (1996) Low oxygen atmosphere initially increases the survival and multiplication of putative goat embryonic stem cells. *Theriogenology* **45:** 237.

All references are incorporated herein by reference.

CLAIMS:

1. A method of improving the developmental ability and quality of *in vitro* cultured pre-implantation embryos comprising the steps:

a) culturing pre-implantation embryos *in vitro* to the pre-compaction or pre-blastulation stage;

b) reducing the capacity of the embryos to generate ATP by oxidative phosphorylation either by partially reducing the oxygen content of the culture atmosphere or by the use of one or more selective oxidative phosphorylation inhibitors and/or uncouplers;

c) culturing the embryos to the compaction or blastocyst stage; and

d) assessing the developmental ability and quality of the embryos for suitability for implantation.

2. A method of improving the viability of an *in vitro* pure culture of embryonic-derived cells comprising the steps:

(a) culturing said cells *in vitro*;

(b) reducing the capacity of the cells to generate ATP by oxidative phosphorylation either by partially reducing the oxygen content of the culture atmosphere or by the use of one or more selective oxidative phosphorylation inhibitors and/or uncouplers;

(c) assessing the viability of said cells for use in nuclear transfer or for any other suitable use.

3. A method as claimed in claim 2, wherein the embryonic cells are embryonic stem cells.

4. A method as claimed in claim 1, wherein the embryos of step a) are 1- or 2- cell early stage embryos.

5. A method as claimed in claim 1, wherein the embryos of step a) are cultured for 4 days to reach pre-compaction or pre-blastulation stage in know culture media under a humidified atmosphere comprising 5 to 7% CO_2, 5 to 7% oxygen and the balance made up of nitrogen.

6. A method as claimed in claim 1 or 2, wherein the oxygen of the atmosphere of the cultured embryos or cultured cells is varied at step b) from 0% to 7%, with the difference in oxygen content being replaced by nitrogen.

7. A method as claimed in claim 1, wherein the embryos of step c) are useful as a source of embryonic stem cells.

8. A method as claimed in claim 1 or 2, wherein the energy substrates are carbohydrates, carboxylic acids and amino acids.

9. A method as claimed in claim 1 or 2, wherein the one or more oxidative phosphorylation inhibitors are selected from the group of known oxidative phosphorylation inhibitors, including sodium azide (NaN_3), cyanide, rotenone and antimycin A, or from any other group of compounds which act as inhibitors of oxidative phosphorylation.

10. A method as claimed in claim 1 or 2, wherein the one or more oxidative phosphorylation uncouplers are selected from the group of known oxidative phosphorylation uncouplers, including 2,4-dinitrophenol (DNP) and carbonylcyanide p-trifluoro-methyoxyphenylhydrazone (FCCP) or from any other group of compounds which act as uncouplers of oxidative phosphorylation from electron transport.

11. A method as claimed in claim 9 or 10, wherein the oxidative phosphorylation inhibitor and/or uncoupler are present in the culture media at a concentration of from 0.001 to 1000μM.

12. A method as claimed in claim 11, wherein the oxidative phosphorylation and/or uncouplers are present in the culture media at a concentration from 1.0 to 250μM.

13. A method as claimed in claim 11, wherein the oxidative phosphorylation and/or uncouplers are present in the culture media at a concentration from 1.0 - 100 μM.

14. A method as claimed in claim 11, wherein the oxidative phosphorylation and/or uncouplers are present in the culture media at a concentration from 1.0 to 20μM.

15. A method as claimed in claim 5 wherein the Day 4 to 5 (3 to 4 days of culture) embryos of step b) are cultured at step c) for 2 to 3 days to reach the compaction or blastocyst stage and assessed at step d) at Day 7 of development.

16. A method as claimed in claim 1 or 2, wherein the pre-implantation embryos or embryonic-derived cells are selected from the group of mammals consisting of cows, pigs, sheep, humans, horses, goats and other domestic animals.

17. A method as claimed in claim 16, wherein the pre-implantation embryos or embryonic-derived cells are cattle embryos or cattle cells.

18. A method as claimed in claim 1 or 2, wherein the pre-implantation embryos or embryonic-derived cells have been obtained from superovulation and standard collection procedures in fertilised mammals, from IVF procedures or *in vitro* nuclear transfer (of either somatic cells or embryonic or embryonic-derived cells) procedures and may have been pre-frozen.

19. An embryo produced by the method of any one of claims 1-18.

20. An embryo as claimed in claim 19 when transferred to a recipient female mammal.

21. An embryo as claimed in claim 19, maintained *in vitro* culture and retaining viability for use in embryo transfer, IVF and/or genetic manipulation, or for use as a source of embryonic cells for nuclear transfer or for embryonic stem cell production.

22. An embryo as claimed in claim 19 when stored or frozen prior to embryo transfer or other manipulation.

23. A culture medium for improving the developmental ability and quality of pre-implantation embryos comprising at least one oxidative phosphorylation inhibitor and/or oxidative phosphorylation uncoupler wherein the inhibitor is selected from the group consisting of sodium azide, cyanide, rotenone and antimycin A, and any other group of compounds which act as inhibitors of oxidative phosphorylation, and the oxidative phosphorylation uncoupler is selected from the group consisting of 2,4-dinitrophenol (DNP), carbonylcyanide p-trifluoro-methoxyphenylhydrazone (FCCP) or any other group of compounds which act as uncouplers of oxidative phosphorylation from electron transport in combination with an animal (eg mammalian) embryo maintaining medium.

24. A culture medium as claimed in claim 23, wherein the oxidative phosphorylation inhibitor and/or uncoupler may be present in the culture medium at a concentration of from 0.001 to 1000μM.

25. A culture medium as claimed in claim 24, wherein the inhibitor and/or uncoupler is present at a concentration of from 1.0 to 250μM.

26. A culture medium as claimed in claim 24, wherein the inhibitor and/or uncoupler is present at a concentration of from 1.0 - 100μM.

27. A culture medium as claimed in claim 24, wherein the inhibitor and/or uncoupler is present at a concentration of from 1.0 to 20μM.

28. A culture medium as claimed in claim 23, wherein the animal embryo maintaining medium comprises a commercially available medium, and it is to this medium to which is added the oxidative phosphorylation inhibitors and/or uncouplers.

29. A culture medium as claimed in claim 28, wherein the commercially available medium comprises a medium for culturing, storing, manipulating and/or freezing pre-implantation embryos.

30. A kit for improving the developmental ability and quality of *in vitro* cultured pre-implantation embryos comprising:

a) commercially available medium suitable for culturing pre-implantation embryos to blastulation and/or compaction;
b) one or more oxidative phosphorylation inhibitors and/or uncouplers in an amount sufficient to enhance the developmental ability and quality of the pre-implantation embryos;

wherein the one or more oxidative phosphorylation inhibitors and/or uncouplers is added to the medium when embryos are at the pre-compaction and pre-blastulation stage.

31. A kit as claimed in claim 30, wherein the one or more oxidative phosphorylation inhibitors are selected from the group consisting of known inhibitors including sodium azide, cyanide, rotenone and antimycin A and from any other group of compounds which act as an inhibitor of oxidative phosphorylation.

32. A kit as claimed in claim 30, wherein the one or more oxidative phosphorylation uncouplers are selected from the group consisting of 2,4-dinitrophenol (DNP), carbonylcyanide p-trifluoro-methoxyphenylhydrazone (FCCP) and from any other group of compounds which act as an uncoupler of oxidative phosphorylation from electron transport.

33. A kit as claimed in claim 30, wherein the amount of the one or more oxidative phosphorylation inhibitors and/or uncouplers added to the culture medium is sufficient to give a concentration of oxidative phosphorylation inhibitor and/or uncoupler in the culture medium of from 0.001 to 1000μM.

34. A kit as claimed in claim 33, wherein the amount of the one or more oxidative phosphorylation inhibitors and/or uncouplers added to the culture medium is sufficient to give a concentration of oxidative phosphorylation inhibitor and/or uncoupler in the culture medium of from 1.0 to 250μM.

35. A kit as claimed in claim 33, wherein the amount of the one or more oxidative phosphorylation inhibitors and/or uncouplers added to the culture medium is sufficient to give a concentration of oxidative phosphorylation inhibitor and/or uncoupler in the culture medium of from 1.0-100μM.

36. A kit as claimed in claim 33, wherein the amount of the one or more oxidative phosphorylation inhibitors and/or uncouplers added to the culture medium is sufficient to give a concentration of oxidative phosphorylation inhibitor and/or uncoupler in the culture medium of from 1-20μM.

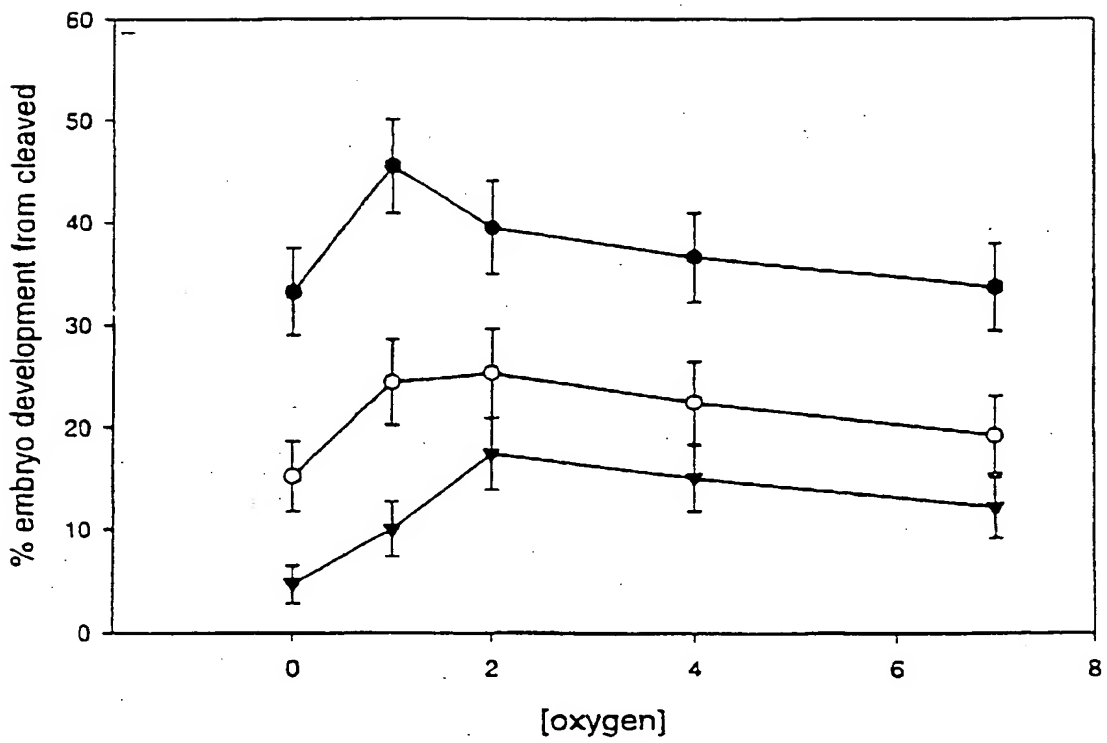

FIG 1

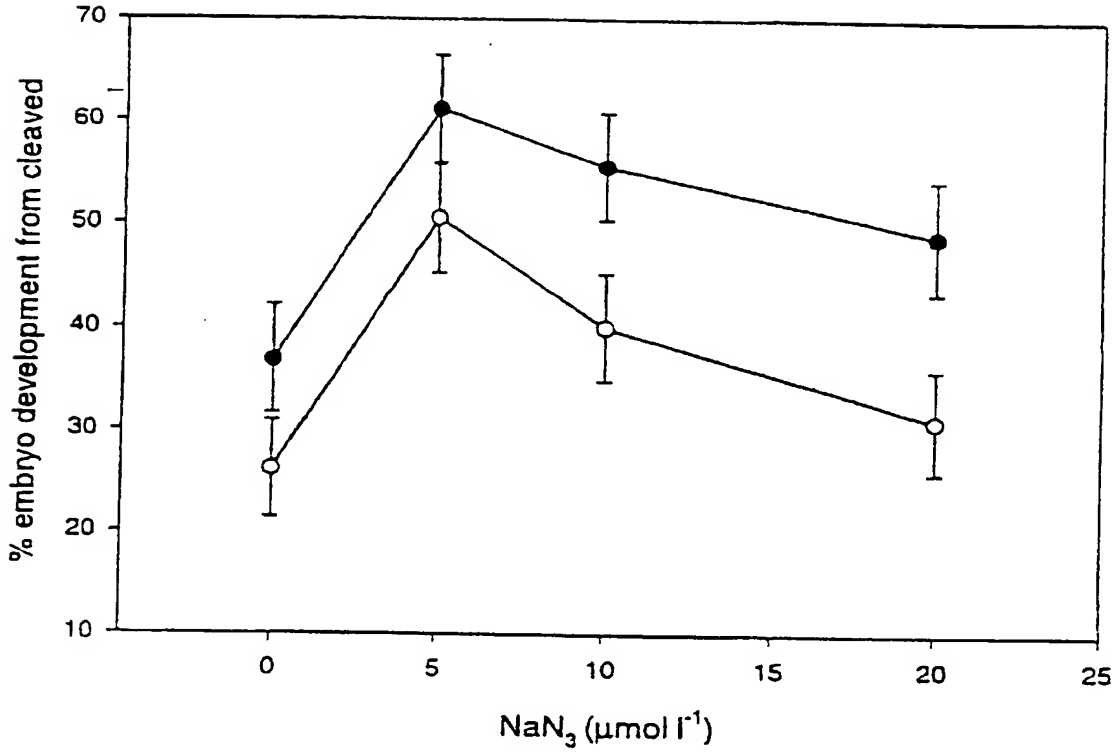

FIG 2

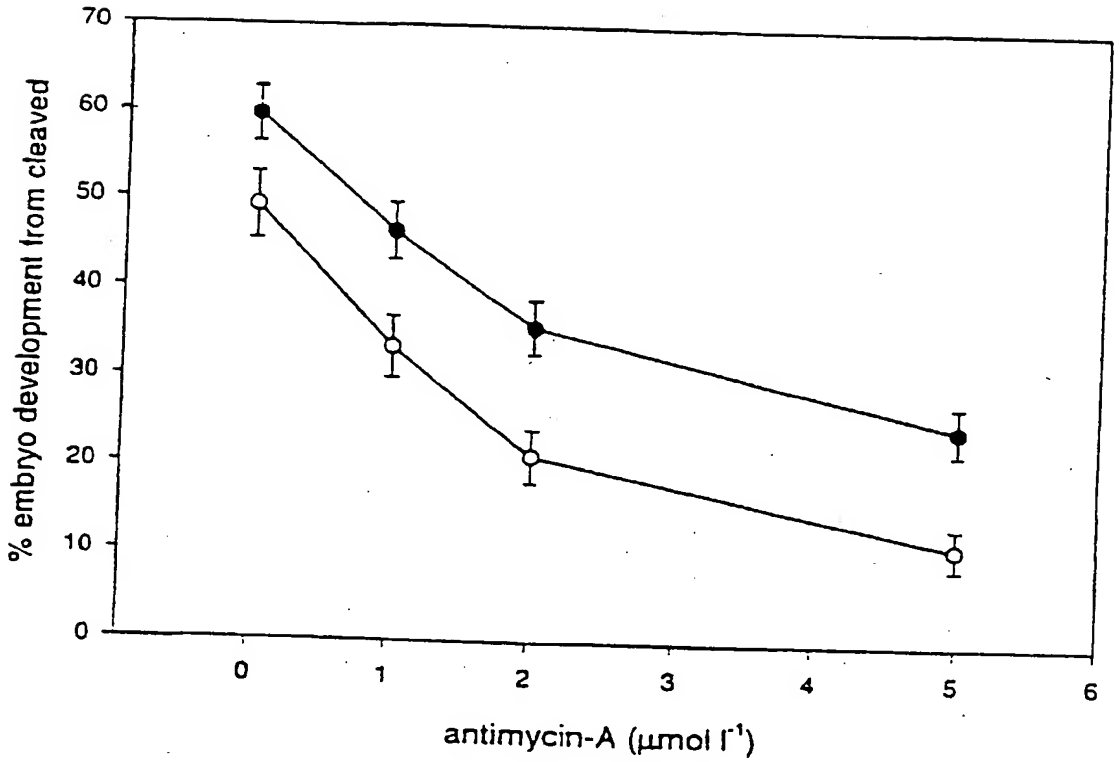

FIG 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ99/00225

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: A61B 17/435; A61D 19/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: AS ABOVE

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SEE ELECTRONIC DATABASES (BELOW)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Medline, WPID, Chem Abs;
Keywords : embryo, oxidative phosphorylation, embryonic()stem()cell, cultur?, uncoupl?, inhibit?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Brison D R; Leese H J "Blastocoel cavity formation by preimplantation rat embryos in the presence of cyanide and other inhibitors of oxidative phophorylation" Journal of Reproduction and Fertility, (1994 Jul) 101 (2) 305-9 Whole document	1-14, 18-20, 23-36
X A	Kane M T; Buckley N J "The effects of inhibitors of energy metabolism on the growth of one-cell rabbit ova to blastocysts in vitro" Journal of Reproduction and Fertility, (1977 Mar) 49 (2) 261-6 See page 261-page 262, line Whole Document	23-36 1-22

[X] Further documents are listed in the continuation of Box C

[ ] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 March 2000	- 8 MAR 2000
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **MR LEIGH R. TRISTRAM** Telephone No.: (02) 6283 2075

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ99/00225

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Ginsbeg L; Hillman N "Shifts in ATP synthesis during preimplantation stages of mouse embryos" Journal of Reproduction and Fertility, (1975 Apr) 43 (1) 83-90 Whole Document	1-36